Thus, all soluble ligand interactions are assumed to have identical affinities, as are all immobilized ligand interactions; the respective microscopic dissociation constants thus are defined simply as K_L' and $K_{\overline{LM}}'$. Given this scheme and the above assumptions, an equation describing the chromatographic elution behavior of bivalent protein on multivalent immobilized ligand matrix is derived, analogously to the previous derivation for the univalent case (Dunn & Chaiken, 1974), as follows.

For liquid chromatography,

$$D = (V - V_m)/V_S \quad \text{(b)}$$

where D is the chromatographic distribution coefficient for the gel, V = elution volume under interacting conditions, V_m = mobile phase volume (outside gel), and V_s = stationary phase volume (inside gel). Also, for the case wherein soluble species can move freely between the inside and outside of the gel,

$$D = ([P_2] + [P_2L \text{ pool}] + [P_2L_2] + [P_2\overline{LM} \text{ pool}] + [P_2\overline{LM}_2] + [P_2L\ \overline{LM} \text{ pool}])/([P_2] + [P_2L \text{ pool}] + [P_2L_2]) \quad \text{(c)}$$

By (1) equating the expressions of D from eq b and c, (2) using the definition

$$D_0 = (V_0 - V_m)/V_S \quad \text{(d)}$$

where V_0 = elution volume of protein when no interaction occurs, and (3) substituting the definitions of K_L' and $K_{\overline{LM}}'$ in the original scheme, one obtains eq e.

$$V = (V_0 - V_m)\left[\left[1 + 2\frac{[L]}{K_L'} + \left(\frac{[L]}{K_L'}\right)^2 + 2\frac{[\overline{LM}]}{K_{\overline{LM}}'} + \left(\frac{[\overline{LM}]}{K_{\overline{LM}}'}\right)^2 + 2\frac{[L][\overline{LM}]}{K_L'K_{\overline{LM}}'}\right] \Big/ \left[1 + 2\frac{[L]}{K_L'} + \left(\frac{[L]}{K_L'}\right)^2\right]\right] + V_m \quad \text{(e)}$$

Rearrangement of eq e leads directly to eq 2 of the main paper; and when [L] = 0, eq 4 of the main paper is obtained. In addition, it should be noted that, when all bivalent interactions are ignored, eq e reduces to the formulation derived earlier for the univalent case, namely, eq 1.

Equation e, or more directly the rearranged eq 2 of the main paper, has been found to adequately describe the data for the competitive elution of IgA monomer on phosphorylcholine–Sepharose. The fitted curve obtained for the high concentration affinity matrix is shown in Figure A1. For comparison, the straight line is shown in Figure A1 that would be obtained if the protein bound only monovalently but with the same microscopic affinities for bound and free ligand.

References

Chaiken, I. M., & Taylor, H. C. (1976) *J. Biol. Chem. 251*, 2044–2048.

Dunn, B. M., & Chaiken, I. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2382–2385.

Dunn, D. M., & Chaiken, I. M. (1975) *Biochemistry 14*, 2343–2349.

Klotz, I. M. (1953) in *Proteins 1B*, 727–806.

Rodbard, D., & Bertino, R. E. (1973) *Adv. Exp. Med. Biol. 36*, 327–341.

Purification and Characterization of RNA Polymerase II Resistant to α-Amanitin from the Mushroom *Agaricus bisporus*†

Allan C. Vaisius and Paul A. Horgen*

ABSTRACT: The DNA-dependent RNA polymerase II or B (ribonucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) from the mushroom *Agaricus bisporus* has been purified to apparent homogeneity. The purification procedures involve precipitation with polyethylenimine, selective elution of RNA polymerase II from the polyethylenimine precipitate, ammonium sulfate fractionation, DEAE-cellulose chromatography, CM-cellulose chromatography, and exclusion chromatography on Bio-Gel A-1.5M. With this procedure 11 mg of RNA polymerase II is recovered from 1.5 kg of mushroom tissue. RNA polymerase II from *Agaricus bisporus* has 12 subunits with the following molecular weights: 182000, 140000, 89000, 69000, 53000, 41000, 37000, 31000, 29000, 25000, 19000, and 16500. Purified RNA polymerase II from *Agaricus bisporous* was half-maximally inhibited by the mushroom toxin α-amanitin at a concentration of 6.5 μg/mL (7×10^{-6} M), which is 650-fold more resistant than mammalian RNA polymerases II. The apparent K_i for the α-amanitin–RNA polymerase complex was estimated to be 12×10^{-6} M. The activity of purified RNA polymerase II from the mushroom was quite typical of other eukaryotic RNA polymerases II with regard to template preference, salt optima, and divalent metal cation optima.

The cytoxin, α-amanitin, is a bicyclic octapeptide occurring in high concentrations in deadly poisonous mushrooms of the genera *Amanita* (Wieland & Wieland, 1972), *Galerina* (Tyler & Smith, 1963), *Conocybe* (Brady et al., 1974), and *Lepiota* (Gérault & Girre, 1975). In addition, Faulstich & Cochet-Meilhac (1976), using very sensitive bioassays, suggest that most mushrooms may contain at least very low concentrations of the amatoxins. The primary cytopathogenicity of the amatoxins is the inhibition of RNA synthesis (Stirpe

† From the Department of Botany and The North American Poison Mushroom Research Centre, University of Toronto, Erindale Campus, Mississauga, Ontario, L5L IC6 Canada. *Received August 31, 1978.* This work was supported in part by National Research Council of Canada Grant A6938.

0006-2960/79/0418-0795$01.00/0

& Fiume, 1967; Kedinger et al., 1970; Lindell et al., 1970).

α-Amanitin is routinely used as a differential inhibitor of the three multiple forms of eukaryotic DNA-dependent RNA polymerase (ribonucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6). In general, RNA polymerase I or A activity is insensitive to α-amanitin, whereas RNA polymerase II or B activity is potently inhibited by α-amanitin, and RNA polymerase III or C activity exhibits moderate sensitivity to α-amanitin (Chambon, 1975; Roeder, 1976). Also, specific inhibition with α-amanitin has been utilized to demonstrate that the different forms of RNA polymerase synthesize different classes of RNA (Chambon, 1975; Roeder, 1976).

The development of several mutant cell lines with α-amanitin-resistant RNA polymerase II activity has been an important tool for studying the regulation of RNA polymerases (Chan et al., 1972; Amati et al., 1975; Ingles et al., 1976; Somers et al., 1975a,b; Wulf & Bautz, 1976; Buchwald & Ingles, 1976; Bryant et al., 1977). Several different lines of evidence indicate that these mutations may involve a structural alteration of RNA polymerase II: (a) there is a measurable decrease in the affinity of the enzyme to α-amanitin (Ingles et al., 1976); (b) there can be coexpression of wild-type sensitive and resistant forms of RNA polymerase II for cultures grown in either the presence or absence of α-amanitin (Somers et al., 1975 a,b; Wulf & Bautz, 1976); (c) there is an increase in the thermolability of RNA polymerase II for the CHO cell line which is α-amanitin resistant (Lobban et al., 1976); (d) there can be an in vitro difference in template preference between the wild-type enzyme and the α-amanitin-resistant form (Bryant et al., 1977). The physical nature of the structural alteration for any RNA polymerase II, however, has not yet been reported. A primary limitation on such structural studies is the need for considerable quantities of highly purified α-amanitin-resistant RNA polymerase II.

RNA polymerases II are extremely complex oligomeric enzymes having two high molecular weight polypeptides associated with several smaller polypeptides. The putative subunit composition of RNA polymerase II is quite similar for all organisms studied thus far, and in this regard the characteristic subunit patterns are perhaps the most definitive method used to differentiate the multiple forms of RNA polymerase (Roeder, 1976; Chambon, 1975). Because of the size and complexity of the enzymes, a determination of the primary structure of RNA polymerases will be an arduous task. However, the interaction between α-amanitin and RNA polymerase II may prove to be important in elucidating certain relationships of structure to function for this enzyme, and to this end, α-amanitin-resistant RNA polymerase II may be quite useful for comparative analysis. Thus far, it has been shown that the α-amanitin-resistant RNA polymerase II from the CHO cell line retains the 140000-dalton subunit (Lobban et al., 1976), which is significant in so far as it has been shown that a subunit of similar molecular weight is the α-amanitin receptor site for calf thymus RNA polymerase II (Brodner & Wieland, 1976). It should be noted that RNA polymerase I from yeast does not have a subunit of 140000 daltons and RNA polymerases III from other eukaryotes have a subunit of slightly lower molecular weight, but nonetheless they too are inhibited by α-amanitin. However, in both of these cases the inhibition by α-amanitin is at higher concentrations than those necessary for the most resistant forms of RNA polymerase II (Valenzuela et al., 1976; Schultz & Hall, 1976; Somers et al., 1975a,b).

In this paper, we report the purification of RNA polymerase II from the edible mushroom *Agaricus bisporus*. The purification procedures modified from Jendrisak & Burgess (1975) involve fractionation with polyethylenimine and ion-exchange chromatography. RNA polymerase II from *Agaricus bisporus* shows only moderate sensitivity to α-amanitin, but nonetheless has other characteristics typical of RNA polymerase II from higher eukaryotes as well as a similar subunit composition. A preliminary report of these findings was given earlier (Vaisius et al., 1977).

Experimental Procedures

Organism. Mushrooms are cultivated *Agaricus bisporus* (Lange) Imbach from the Leaver Mushroom Co., Mississauga, Ontario. The mushrooms were always freshly picked on the day of an experiment, and the fruiting bodies were not cut until immediately prior to homogenization.

Chemicals. Calf thymus DNA (Type I), nucleoside triphosphates, dithiothreitol, phenylmethanesulfonyl fluoride, and 2-mercaptoethanol were purchased from Sigma. Tritium-labeled UTP (sp act. 36.7 Ci/mmol) was purchased from New England Nuclear. Enzyme-grade ammonium sulfate was obtained from Schwarz/Mann. Polyethylenimine (50%) and Brij 35 were purchased from BDH. DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were from Whatman, and Bio-Gel A-1.5M was from Bio-Rad. Radioactive samples were suspended in PCS cocktail purchased from Amersham/Searle.

Acrylamide, methylenebis(acylamide), ammonium persulfate, tetraethylmethylenediamine, Coomassie brilliant blue R250, and sodium dodecyl sulfate were purchased from Bio-Rad. Protein markers for molecular weight determinations were myosin (200000), β-galactosidase (130000), phosphorylase B (94000), bovine serum albumin (68000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (21000), and lysozyme (14300) from Bio-Rad and *Escherichia coli* RNA polymerase from Miles.

Spectroscopically pure manganese chloride and magnesium chloride were obtained from Johnson Matthey Chemicals.

Solutions. All buffers were prepared with glass doubly distilled water. Buffers were freshly prepared from the following stock solutions: 2 M Tris-HCl (pH 7.9), 0.1 M EDTA (adjusted to pH 7.9 with NaOH), 2 M $(NH_4)_2SO_4$ (adjusted to pH 7.9 with NH_4OH), and 0.1 M phenylmethanesulfonyl fluoride in absolute ethanol. Solid dithiothreitol or 2-mercaptoethanol was added immediately prior to use. A 5% (v/v) solution of polyethylenimine was prepared in 0.1 M Tris-HCl (adjusted to pH 7.9).

The homogenization buffer (H buffer) was 0.1 M Tris-HCl (pH 7.9), 0.05 M $(NH_4)_2SO_4$, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, and 1.0 mM phenylmethanesulfonyl fluoride. The first polyethylenimine elution buffer (0.06 M PE buffer) was the same as H buffer except that it was 0.06 M $(NH_4)_2SO_4$. The second polyethylenimine elution buffer (0.25 M PE buffer) was the same as H buffer except that it was 0.25 M $(NH_4)_2SO_4$ and the phenylmethanesulfonyl fluoride was omitted.

The chromatography buffer (C buffer) was 25% (v/v) ethanediol, 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA, and 1.0 mM dithiothreitol. The designation of molarity onto a C buffer is an indication of the $(NH_4)_2SO_4$ concentration, e.g., 0.05 M C buffer.

Salt Determination. The ammonium sulfate concentration was determined as a function of specific conductance. Conductivity measurements were made with a Wheatstone bridge (Beckman conductance bridge, no. RC-16C), and the standard curves were calculated from conductometric titrations

of the appropriate buffer systems.

Preparation of Ion-Exchange Resins. Precycled DEAE-cellulose (Whatman DE-52) was suspended in five volumes of 0.5 M C buffer and allowed to stand overnight. The resin was then successively washed five times, each time with two volumes of 0.15 M C buffer. The resin was then degassed and packed into a 4.5 × 35 cm column and washed with 0.15 M C buffer until equilibration as indicated by pH and conductivity measurements.

CM-cellulose (Whatman CM-52) was prepared in a similar fashion except that the resin was equilibrated to 0.075 M C buffer and then was packed into a 5 × 10 cm column.

Protein Determinations. Protein concentrations were estimated using the Bio-Rad protein assay with bovine γ-globulin as a standard.

Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed under both denaturing and nondenaturing conditions. For electrophoresis under denaturing conditions, sodium dodecyl sulfate-polyacrylamide gels were prepared, using the buffer systems described by Laemmli (1970). Slab gels were cast with a 1-cm stacking gel and a 20-cm separating gel. A sample was usually run on gels of different acrylamide concentrations ranging from a 7.5 × 1 separating gel with a 5 × 2 stacking gel to a 15 × 1 separating gel with a 7.5 × 1 stacking gel, i.e., by use of the $T \times C$ notation of Hjertên (1962) to designate the total concentration of the acrylamide monomers (T) as well as the percent of the cross-linking agent (C) with respect to T. The enzyme samples were dialyzed overnight against 1000 volumes of sample buffer [0.0625 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate, and 1% (v/v) 2-mercaptoethanol]. Electrophoresis was carried out at an applied current of 0.5 mA/cm^2. Immediately upon completion of electrophoresis, the gels were severed at the dye front, and then the polypeptides were fixed in place with 12.5% (w/v) trichloracetic acid for 1 h. The polypeptides were then stained in 10% (v/v) acetic acid, 25% (v/v) methanol, and 0.1% (w/v) Coomassie brilliant blue R250 for at least 8 h. The gels were destained in 25% (v/v) methanol and 10% (v/v) acetic acid until the bands appeared, and then they were transferred to 7% (v/v) acetic acid and allowed to completely destain. The molecular weights of the peptide bands were estimated statistically from a standard curve determined through least-squares regression on a programmed calculator. The standard curve was made using the relationship R_f vs. log M_r as described by Shapiro et al. (1967).

For electrophoresis under nondenaturing conditions, the polyacrylamide gels were prepared using the buffer systems described by Davis (1964). The gels were cast in 0.7 × 12 cm cylinders with a 1-cm stacking gel of 3 × 1 acrylamide and a 9-cm separating gel of either 5 × 1, 6 × 1, or 7 × 1 acrylamide. The cylindrical gels were stained as described by Chrambach et al. (1967) and scanned at 550 nm using a Gilford Linear Transport attached to a Gilford spectrophotometer.

RNA Polymerase Assay. All glassware used in the assay procedures were siliconized prior to use, and the assay tubes were used but once.

The standard assay solution was 0.25 mL, having 10 mM Tris-HCl (pH 7.9); 5% (v/v) ethanediol or 5% (v/v) glycerol; 10 μM EDTA; 2 mM dithiothreitol; 4 mM $MgCl_2$; 2 mM $MnCl_2$; 75 mM $(NH_4)_2SO_4$; 40 μM each of GTP, CTP, and ATP; 1 nmol of [^{3}H]UTP (sp act. 1 μCi/nmol); and 100 μg of denatured calf thymus DNA. The assay solution was incubated for 15 min at 30 °C. The reaction was terminated, and the RNA product was precipitated with the addition of 2 mL of an ice-cold solution of 10% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate. After standing for 15 min on ice, the precipitates were collected on glass fiber filters (Whatman GF/C), washed five times in succession with 5 mL of ice-cold 2.5% (w/v) trichloroacetic acid, and rinsed with 5 mL of 95% ethanol. Radioactivity was counted in PCS–xylene (2:1) on a Beckman LS-150 scintillation spectrometer.

One unit of RNA polymerase activity is defined as the incorporation of 10 pmol of labeled UMP into acid-precipitable form per 15 min at 30 °C. The incorporation level of a reaction mixture incubated without enzyme was subtracted from all activity determinations. Routinely all activity fractions were assayed for DNA dependency and the dependency upon all four nucleoside triphosphates.

Calf thymus DNA was allowed to hydrate in a sterilized solution containing 10 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, and 10 mM $(NH_4)_2SO_4$. The DNA was denatured by gradually bringing the solution to 100 °C in a block heater and then immediately cooling the solution in an ice–water bath.

Concentrated α-amanitin was kept in absolute methanol and serial dilutions into glass doubly distilled water were prepared immediately prior to use. The α-amanitin concentration was determined using the molar extinction coefficient (ϵ = 11 900) at the absorption maxima (λ_{max} = 305 nm) as determined by Gebert et al. (1967). In a titration with α-amanitin, the highest concentration of methanol in any assay is 5% (v/v). A parallel control assay without the toxin but with 5% (v/v) methanol showed no inhibition relative to the standard assay.

RNA Polymerase II Purification. The purification procedures for RNA polymerase II are modified from Jendrisak & Burgess (1975). All operations were carried out in a cold room at 4 °C.

Immediately prior to homogenization, mushroom tissue was cut up into relatively uniform pieces to facilitate homogenization. The mushrooms were homogenized in 1 mL of H buffer/1 g of tissue in a Waring blender for 1 min at low speed. The homogenate was centrifuged at 10000g for 15 min in a Sorvall HB-4 rotor.

The supernatant was filtered through miracloth and adjusted to pH 7.9 if necessary. To this was added 75 μL/mL of 5% (v/v) polyethylenimine. The solution was kept stirring for 5 min and centrifuged as described above. The pellets were suspended in 1 L of 0.06 M PE buffer (i.e., for 1.5 kg of mushroom tissue) using a Willems Polytron Homogenizer (Brinkmann Instruments) at low speed for 1 min. The suspension was centrifuged at 10000g for 15 min. The pellets were suspended in 750 mL of 0.25 M PE buffer as described. The pH of the suspension solution was carefully monitored and adjusted to 7.9 if necessary. The suspension was centrifuged at 10000g for 15 min and the pellets were discarded. Protein in the supernatant fraction was precipitated with 0.25 g/mL of solid ammonium sulfate. After it was certain that all the salt was dissolved, the solution was packed in ice and allowed to stand overnight without loss of activity. The ammonium sulfate precipitate was centrifuged at 15000g for 45 min. The pellets were suspended in 60 mL of C buffer containing 0.1% (v/v) Brij 35. This suspension was centrifuged at 10000g for 15 min to clear the solution of any residual polyethylenimine. The supernatant was further diluted with C buffer so that the conductivity was identical with that of the 0.15 M C buffer.

The ammonium sulfate precipitate fraction was applied at a flow rate of 50 mL/h to a DEAE-cellulose column (250 mL) equilibrated to 0.15 M C buffer. The column was washed with

Table I: Summary of Purification of RNA Polymerase II from the Mushroom *Agaricus bisporus*

fraction	volume (mL)	protein (mg)	total act. (units)	sp act. (units/mg)[a]	purification factor	yield (%)
crude supernatant	2740	16981	4226	0.249	1	100
polyethylenimine eluant with 0.25 M PE buffer	750	1377	4007	2.9	11.6	95
$(NH_4)_2SO_4$ fractionation	170	460	3902	8.5	34.1	92
DEAE-cellulose peak	59	40.8	3506	85.9	344.9	83
CM-cellulose peak	22.5	15.9	3421	215.0	863.5	81
Bio-Gel A-1.5M peak	7.5	11	3244	294.0	1180.7	77

[a] One unit of RNA polymerase activity is defined as the incorporation of 10 pmol of labeled UMP into acid-precipitable form per 15 min at 30 °C.

275 mL of 0.15 M C buffer. RNA polymerase was eluted from the column with a 225 mL step gradient of 0.25 M C buffer, which was then followed by 100 mL step elution with 0.5 M C buffer.

Fractions from the peak of RNA polymerase activity were concentrated by precipitation with 0.3 g/mL of solid ammonium sulfate and centrifuged at 35 000 rpm (94600*g* at the tube center) for 45 min in a Spinco 42.1 rotor. Each pellet was suspended with 10 mL of C buffer. The pooled sample solutions were further diluted with C buffer so that the conductivity was identical with that of the 0.075 M C buffer. This sample was applied at a flow rate of 60 mL/h to a CM-cellulose column (125 mL) equilibrated with 0.075 M C buffer. The column was washed with 175 mL of 0.075 M C buffer, and the RNA polymerase was recovered in the flow-through fractions. The column was then step eluted with 45 mL of 0.15 M C buffer, which was in turn followed by a step elution with 45 mL of 0.3 M C buffer.

Peak fractions from the CM-cellulose column were pooled, and the protein was precipitated by dialysis against C buffer 65% saturated with ammonium sulfate. The sample solution was centrifuged for 45 min at 40 000 rpm (102900*g* at the tube center) in a Spinco 65 rotor. The pellets were suspended in a gel chromatography buffer which was 25% (v/v) glycerol, 0.05 M Tris-HCl (pH 7.9), 0.05 M $(NH_4)_2SO_4$, 0.1 mM EDTA, and 1.0 mM dithiothreitol. The sample was applied to a 2 × 30 cm column of Bio-Gel A-1.5M equilibrated to the same buffer. The flow rate was 24 mL/h. The peak fraction was brought to 50% (v/v) glycerol and has been kept indefinitely at −70 °C without loss of activity.

Results

Purification Procedures. A summary of RNA polymerase II purification from the mushroom *Agaricus bisporus* is given in Table I. After centrifugation of the crude homogenate, the supernatant solution shows a substantial increase in volume (>50%) over the original volume of the buffer. This dilution from the mushroom extract requires that the concentration of polyethylenimine be 75 μL/mL to precipitate RNA polymerase activity depsite the fact that the total protein concentration is relatively low. Under these conditions, approximately 17% of the protein in the crude homogenate is precipitated.

In washing the polyethylenimine pellets in 0.06 M PE buffer, 721 mg of protein is extracted. In this first wash there are 876 units of RNA polymerase activity with a specific activity of 1.2. This activity is entirely insensitive to 25 μg/mL of α-amanitin. The purification of this fraction will be described in a future communication.

The optimal ionic condition for eluting RNA polymerase II from the polyethylenimine was found to be 0.25 M ammonium sulfate in the PE buffer. A further increase in ionic strength of the elution buffer did not result in an increase in yield. We have found a degree of irregularity in the measurable activity of the crude homogenate, whereas the activity eluted from the polyethylenimine precipitate is reproducible from one experiment to the next. RNA polymerase activity eluted with 0.25 M PE buffer is 75% inhibited by 25 μg/mL of α-amanitin. However, if the first wash with 0.06 M PE buffer is omitted, the RNA polymerase activity in the final elution is much less sensitive to α-amanitin.

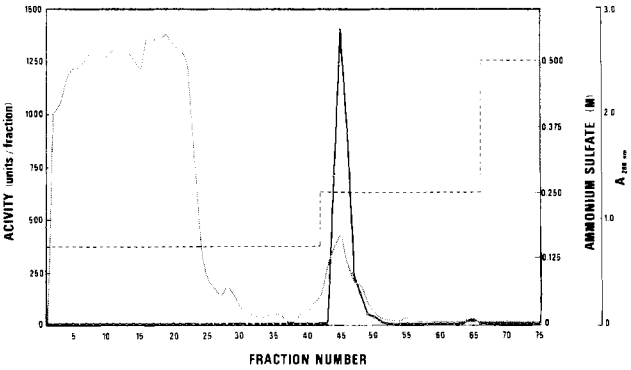

FIGURE 1: DEAE-cellulose chromatography of the resuspended precipitate resulting from the ammonium sulfate fractionation. The applied sample and the DEAE-cellulose column were equilibrated to 0.15 M C buffer as determined by conductivity measurements. The column was step eluted as described in the text. The flow rate was maintained at 50 mL/h. Fractions of 9.5 mL were collected. RNA polymerase activity eluted at 0.25 M $(NH_4)_2SO_4$. Fractions 44–48 were pooled and concentrated as described in the text. RNA polymerase activity (–O–). Ammonium sulfate concentration (- - -). A_{280nm} (···).

Fractionation of the final polyethylenimine eluant by precipitation with 0.25 mg/mL of ammonium sulfate resulted in greater than threefold purification with a 92% recovery of the total activity. Moreover, sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the ammonium sulfate precipitate fraction indicates a substantial decrease in the heterogeneity of polypeptides with this fractionation.

DEAE-cellulose chromatography of the fraction resulting from ammonium sulfate precipitation is shown in Figure 1. This step results in a 10-fold purification of RNA polymerase with 90% recovery. If the DEAE-cellulose column is developed with a linear gradient from 0.075 to 0.4 M C buffer, the peak of RNA polymerase activity elutes at 0.21 M ammonium sulfate. However, elution with a linear gradient does not result in a greater yield of total or specific activity than that achieved with a step elution as shown in Figure 1.

The activity pool from the DEAE-cellulose peak was concentrated as well as equilibrated to 0.075 M C buffer as described and then chromatographed on CM-cellulose as shown in Figure 2. Because RNA polymerase II does not bind to CM-cellulose at this ionic strength, this is a very efficient separation step, resulting in 98% recovery while achieving a further 2.5-fold purification. When analyzed using

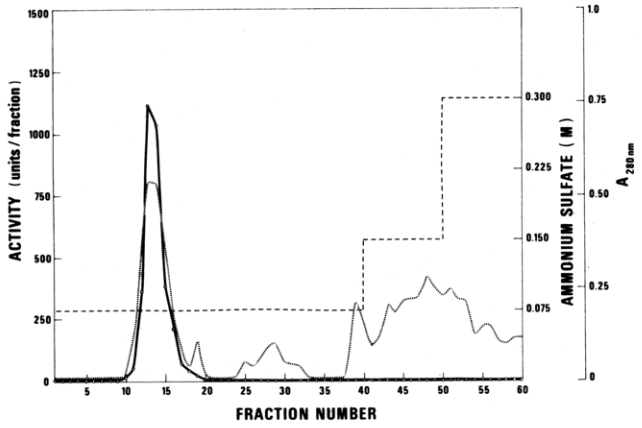

FIGURE 2: CM-cellulose chromatography of the RNA polymerase activity recovered from DEAE-cellulose chromatography. The applied sample and the CM-cellulose were equilibrated to 0.075 M C buffer as determined by conductivity measurements. The column was step eluted as described in the text. The flow rate was maintained at 60 mL/h. Fractions of 4.5 mL were collected. RNA polymerase activity is recovered in the flow-through fractions. Fractions 12–16 were pooled and concentrated as described in the text. RNA polymerase activity (–O–). Ammonium sulfate concentration (---). A_{280nm} (•••).

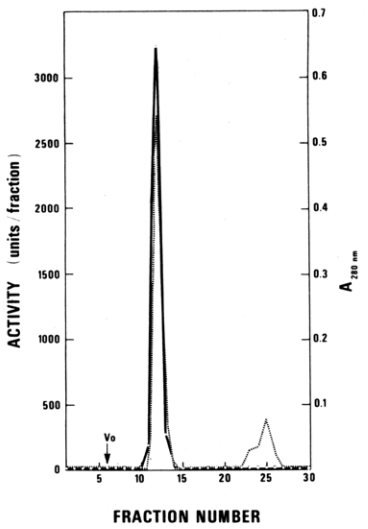

FIGURE 3: Exclusion chromatography on Bio-Gel A-1.5M of the RNA polymerase activity recovered from CM-cellulose chromatography. The applied sample and the Bio-Gel A-1.5M were equilibrated to the gel chromatography buffer described in the text. The flow rate was maintained at 24 mL/h. Fractions of 3.5 mL were collected. The void volume (V_0) was determined with blue dextran in a separate experiment. RNA polymerase activity (–O–). A_{280nm} (•••).

sodium dodecyl sulfate–polyacrylamide gel electrophoresis, it is seen that this fraction has the subunits characteristic of RNA polymerase II with very few contaminating species.

Exclusion chromatography on Bio-Gel A-1.5M is shown in Figure 3. RNA polymerase remains concentrated through this step as the activity is primarily recovered in a single fraction. Recovery of activity in the peak fraction is 95% and the purification is 1.4-fold. The absorbance peak near the end of the column represents protein which has been separated from the RNA polymerase activity as substantiated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Polyacrylamide Gel Elecrophoresis under Nondenaturing Conditions. The purified RNA polymerase was analyzed through polyacrylamide gel electrophoresis as shown in Figure 4. The gels were heavily loaded with protein (90 μg) so as to be more amenable to the visualization of any contaminating proteins. After the gels were stained with Coomassie brilliant blue R250 and then destained, only one prominent protein

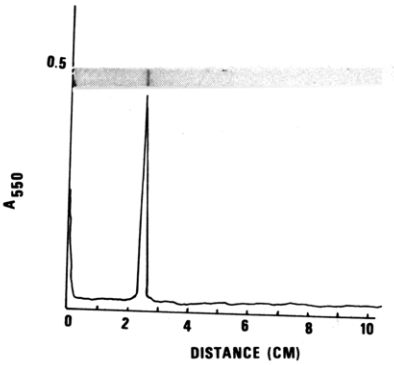

FIGURE 4: Polyacrylamide gel electrophoresis under nondenaturing conditions of purified RNA polymerase II (90 μg) as described in the text. The gel shown in the figure is 6 × 1 acrylamide in the Hjertén (1962) notation.

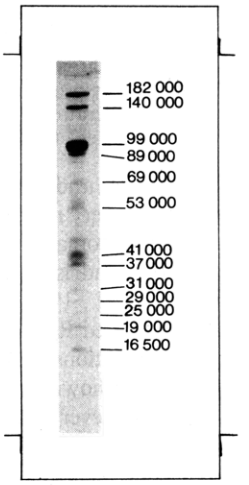

FIGURE 5: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified RNA polymerase II as described in the text. The gel shown in the figure has a separating gel of 12.5 × 1 acrylamide by use of the notation of Hjertén (1962). The molecular weights of the subunits were determined from several other gels as described in the text by use of the following marker proteins: myosin (200 000), *E. coli* RNA polymerase β (165 000), *E. coli* RNA polymerase β′ (155 000), β-galactosidase (130 000), *E. coli* RNA polymerase σ (95 000), phosphorylase B (94 000), bovine serum albumin (68 000), ovalbumin (43 000), *E. coli* RNA polymerase α (39 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (21 000), and lysozyme (14 300).

band was evident, thus indicating that the purity of the enzyme preparation was nearly homogenous. A single protein band was also obtained with gels cast with concentrations of 5 × 1 and 7 × 1 acrylamide.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The subunit composition of the purified RNA polymerase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described earlier and shown in Figure 5. The enzyme fraction used for subunit molecular weight determinations was that recovered in the peak fraction of Bio-Gel A-1.5M chromatography as minor contaminating species were removed in this purification step. By use of a low applied current over a long separation phase, a very good statistical fit is obtained for the high molecular weight species in gels of 7.5 × 1 and 10 × 1 acrylamide and for the low molecular weight species in gels of 10 × 1, 12.5 × 1, and even 15 × 1 acrylamide. The tentative subunits of RNA polymerase II for *Agaricus bisporus* have the following molecular weights: (a) 182 000, (b) 140 000, (c) 89 000, (d) 69 000, (e) 53 000, (f) 41 000, (g) 37 000, (h) 31 000, (i) 29 000, (j) 25 000, (k) 19 000, and (l) 16 500. There is also a prominently stained band at 99 000 daltons, which may be a contaminating species.

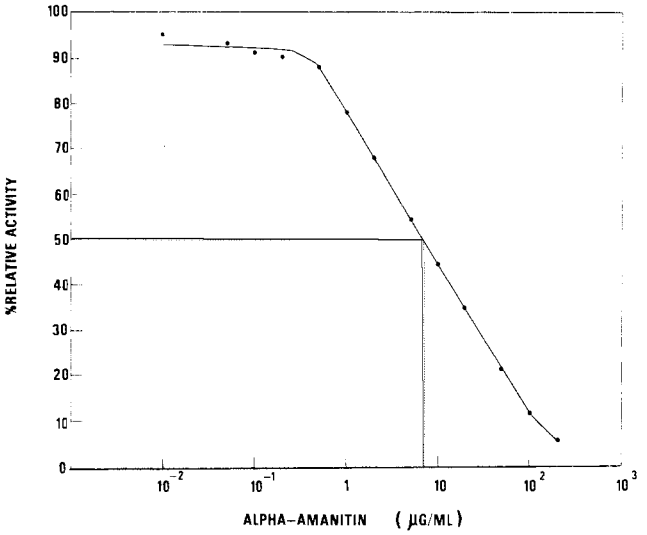

FIGURE 6: Titration of RNA polymerase II from *Agaricus bisporus* with α-amanitin. The standard assay procedures were used as described within the text. The concentration of α-amanitin was determined from the extinction coefficient (Gebert et al., 1967).

Except for the subunits (c), (d), and (e), the subunit composition for *Agaricus bisporus* is very much like that of higher eukaryotes, e.g., mouse plasmacytoma, calf thymus, rat liver, and several species of plants (Jendrisak & Guilfoyle, 1978; Sklar et al., 1975; Kedinger et al., 1974).

α-Amanitin Sensitivity. Purified RNA polymerase II was titrated with increasing concentrations of α-amanitin and the relative activity determined as shown in Figure 6. These results have been reproduced for several enzyme preparations as well as for two different preparations of α-amanitin. RNA polymerase II from *Agaricus bisporus* is half-maximally inhibited by 6.5 μg/mL (7×10^{-6} M) of α-amanitin.

Cochet-Meilhac & Chambon (1974) have shown that the inhibition of calf thymus RNA polymerase II by α-amanitin is noncompetitive and that the ligand binding occurs with one-to-one stoichiometry. From the titration curve in Figure 6, it can be seen that the inhibition of RNA polymerase II from *Agaricus bisporus* by α-amanitin is "pure"; e.g., at a fixed substrate concentration the activity will approach zero at a sufficiently high concentration of inhibitor. At different substrate concentrations, a pure noncompetitive inhibition mechanism predicts that linear plots of 1/activity vs. inhibitor concentration for each of the substrate concentrations will meet at a point on the x intercept which is equal to $-K_i$ (Dixon, 1953). The inhibition constant, K_i, of α-amanitin for RNA polymerase II from *Agaricus bisporus* has been estimated graphically to be 12×10^{-6} M by use of the Dixon plot shown in Figure 7. Direct measurement of ligand binding by use of the membrane filter assay developed by Cochet-Meilhac and Chambon (1974) is not experimentally detectable for RNA polymerases half-maximally inhibited by concentrations greater than 2 μg/mL (Ingles et al., 1976).

Effect of Mn^{2+} and Mg^{2+}. A titration of purified RNA polymerase II with spectroscopically pure Mn^{2+} or Mg^{2+} is shown in Figure 8. RNA polymerase II exhibited strong optimal activity at 2.5 mM Mn^{2+}. Quite surprisingly, activity is only observed in the presence of Mn^{2+}. In fact, evidence indicates that Mg^{2+} may be antagonistic to the optimal activity shown in the presence of spectroscopically pure Mn^{2+} alone. A complete study on the effects of divalent cations will be published elsewhere.

Effect of Ionic Strength. Purified RNA polymerase II was treated with ammonium sulfate as shown in Figure 9. The

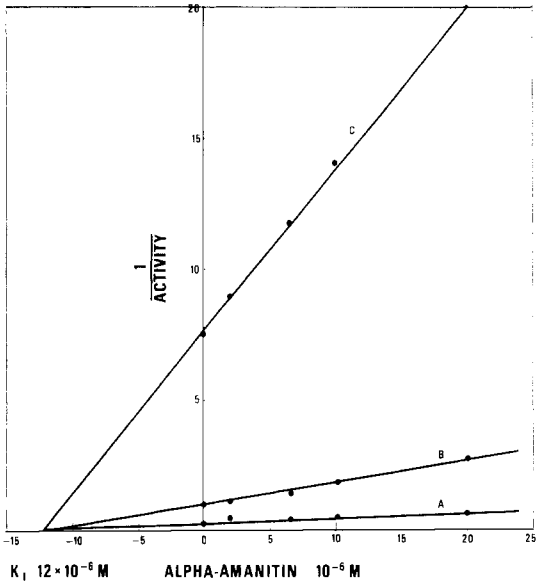

FIGURE 7: Determination of the inhibition constant, K_i, of α-amanitin for the purified RNA polymerase II from the mushroom. The standard assay procedures were adhered to with the following exceptions: $MgCl_2$ was omitted; the concentration of $MnCl_2$ was 2.5 mM; and the concentrations of ATP, CTP, GTP, and UTP were 1.0 nmol each for line A, 0.5 nmol each for line B, and 0.1 nmol each for line C. The best fit lines were determined by use of preprogrammed linear regression analysis. The enzyme concentration was 12 μg per reaction. The concentration of α-amanitin was determined from the extinction coefficient (Gebert et al., 1967).

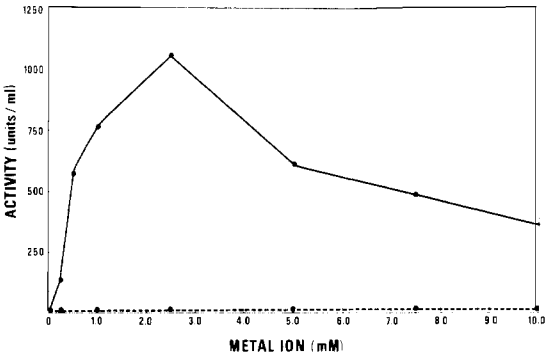

FIGURE 8: Titration of RNA polymerase II from *Agaricus bisporus* with spectroscopically pure $MnCl_2$ (–●–) or $MgCl_2$ (- -●- -). Other than the divalent metal ion concentration, the standard assay procedures were followed.

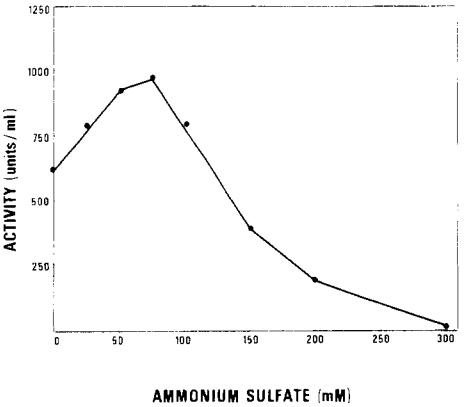

FIGURE 9: Titration of RNA polymerase II from *Agaricus bisporus* with ammonium sulfate. The standard assay procedures were adhered to with the exception of the following modifications to the assay solution: $MgCl_2$ was omitted and the concentration of $MnCl_2$ was 2.5 mM.

purified enzyme exhibits a sharp response to the ionic condition with an optimum at 75 mM $(NH_4)_2SO_4$.

Template Activities. The activity of purified RNA polymerase II was determined using either native DNA or denatured DNA as the template. The assay procedures previously described were adhered to with the exception of the following modifications to the assay solution: $MgCl_2$ was omitted, the concentration of $MnCl_2$ was 2.5 mM, and 50 μg of either native or denatured DNA was used as the template. RNA polymerase II from the mushroom had an activity ratio of 2.2:1 for denatured DNA/native DNA. However, we cannot rule out an artifactual preference for denatured DNA by RNA polymerases II as suggested by Dynan et al. (1977).

Discussion

Purified RNA polymerases II have been characterized and the subunit composition determined for several species of fungi, including *Saccharomyces cerevisiae* (Dēzelēe & Sentenac, 1973; Hildebrandt et al., 1973; Valenzuela et al., 1976), *Physarum polycephalum* (Hildebrandt & Sauer, 1973; Burgess & Burgess, 1974; Gornicki et al., 1974; Smith & Braun, 1978), *Dictyostelium discoideum* (Pong & Loomis, 1973), and *Mucor rouxii* (Young & Whiteley, 1975). This paper is the first report of the purification of RNA polymerase II from a higher basidiomycete. Despite the fact that the total soluble protein content in the crude homogenate is relatively low in comparison to plant and animal sources (Jendrisak & Burgess, 1975; Hodo & Blatti, 1977), mushroom tissue is quite rich in RNA polymerase II. From 1.5 kg of fresh tissue, 11 mg of nearly homogenous RNA polymerase II was obtained with a recovery of 77% of the total activity. The optimal experimental parameters for the selective precipitation of RNA polymerase II by polyethylenimine were determined as suggested by Jendrisak & Burgess (1975). We further enhanced our enzyme purification by optimizing the ionic conditions of the initial wash of the polyethylenimine pellets. This step also separated another RNA polymerase activity insensitive to very high concentrations of α-amanitin. The recovery of total RNA polymerase II activity through DEAE-cellulose chromatography remains quite high at 83%. In determining the most efficient succeeding purification steps, alternate ion-exchange resins such as phosphocellulose and DEAE-Sephadex were used. Chromatography on the alternate resins did not result in a greater purification than that achieved with CM-cellulose, but in all cases the recovery was much lower than that yielded on CM-cellulose. Chromatography on CM-cellulose had the advantage of very efficiently removing any trace contaminants of RNA polymerase I or III as they would have most likely bound to the resin at 0.075 M $(NH_4)_2SO_4$, whereas RNA polymerase II does not bind under these conditions (Schwartz et al., 1974). Exclusion chromatography on Bio-Gel A-1.5M was used as a final purification step to remove possible residual contaminating species of differing molecular weight, i.e., providing that they had not become tightly associated with the RNA polymerase enzyme. The purified RNA polymerase has a specific activity (294 units/mg) very comparable to that reported by Jendrisak & Burgess (1975) for wheat germ RNA polymerase II isolated and assayed with generally similar procedures.

In analyzing the purified enzyme on polyacrylamide gels under nondenaturing conditions, several procedural considerations were made to better visualize any contaminating species which may have been present: (a) a large quantity of protein (90 μg) was applied to the gels, (b) the gels were cast with a long separating phase (10 cm) in cylinders of small diameter (0.7 cm), and (c) samples were run on gels of differing acrylamide concentration. In all cases a single prominent protein band was visualized, thus indicating that RNA polymerase II had been purified to near homogeneity.

The purification of RNA polymerase II from the mushroom may have general evolutionary significance in light of the fact that the enzyme from the mushroom is less sensitive to α-amanitin than any other eukaryotic RNA polymerase II purified thus far. Recently, we reported that RNA polymerase activities in nuclear fractions from *Amanita phalloides* and two other species of mushroom were highly insensitive to α-amanitin when compared to nuclear fractions from the Oomycete, *Achlya ambisexualis*, and rabbit brain (Horgen et al., 1978). Typically, RNA polymerases II from animal cells, insects, or amphibians have been shown to be half-maximally inhibited by α-amanitin concentrations of 0.01–0.05 μg/mL (Roeder, 1976; Chambon, 1975). Recently, it was shown that RNA polymerases II from a wide variety of plants are similarly inhibited by α-amanitin (Jendrisak & Guilfoyle, 1978). The enzyme from yeast is an anomaly as it is half-maximally inhibited at 0.8–1.0 μg/mL (Schultz & Hall, 1976; Valenzuela et al., 1976). The mutant cell lines resistant to α-amanitin are not entirely resistant to the toxin but, rather, much less sensitive to α-amanitin with the most resistant mutant cell line, rat myoblast Ama 102, half-maximally inhibited at 5 μg/mL (Somers et al., 1975a,b). In this paper, we report that RNA polymerase II from the mushroom *Agaricus bisporus* is half-maximally inhibited by α-amanitin at a concentration of 6.5 μg/mL (7×10^{-6} M). From the titration curve in Figure 6, it is seen that the α-amanitin inhibition acts on a single component; i.e., the titration is not biphasic, thus indicating that other contaminating classes of RNA polymerase are not present.

In comparing the association and dissociation rate constants for different amatoxins on the amatoxin–RNA polymerase II complex, Cochet-Meilhac & Chambon (1974) suggest that the in vivo toxicity of the amatoxins is governed by the dissociation rate of the complex. It follows that differences in resistance most likely reflect differences in the dissociation rate of the complex with higher resistance being manifested in a greater K_D. The increase in the dissociation rate results in experimental constraints on the direct measurement of ligand binding because of the shorter half-life of the complex (Morris et al., 1978). However, the inhibition constant, K_i, is experimentally very comparable to the K_D for the amatoxin–RNA polymerase II complex (Cochet-Meilhac & Chambon, 1974), and the inhibition constant as defined by Dixon (1953) actually represents the dissociation constant for the inhibitor–enzyme complex or the inhibitor–enzyme–substrate complex. By use of a Dixon plot (Dixon, 1953), the K_i has been estimated to be 12×10^{-6} M for the complex between α-amanitin and RNA polymerase II from *Agaricus bisporus*. Furthermore, the Dixon plot in Figure 7 substantiates the pure noncompetititive inhibition of the mushroom RNA polymerase II by α-amanitin.

Since the amatoxin binding site for calf thymus RNA polymerase II is the 140 000-dalton subunit (Brodner & Wieland, 1976), it is significant that the much less sensitive enzyme from the mushroom also has a subunit of 140 000 dalton. The differences in sensitivity to α-amanitin may reflect differences in the primary structure of the binding site on the 140 000-dalton subunit or differences in the quaternary structure of the enzyme. In this regard, we are, at present, conducting a much more extensive study on the subunit composition of RNA polymerase II from *Agaricus bisporus*. However, our preliminary results indicate that the enzyme

from the mushroom has a number of subunits with apparent molecular weights quite similar to those subunits with a common molecular weight in a number of plant and animal cells. For example, RNA polymerase class IIB from calf thymus, rat liver, cauliflower, and soybean has a 180 000-dalton subunit (Kedinger et al., 1974; Jendrisak & Guilfoyle, 1978), and the mushroom enzyme has a subunit of 182 000 dalton. RNA polymerases II from mouse plasmacytoma, calf thymus, rat liver, and many other eukaryotes as well as *Agaricus bisporus* have subunits of 140 000 and 16 500 daltons (Kedinger et al., 1974; Schwartz & Roeder, 1975; Jendrisak & Guilfoyle, 1978). RNA polymerase II from the mushroom has a subunit of 25 000 daltons as does calf thymus, rat liver, and several plant species (Kedinger et al., 1974; Jendrisak & Guilfoyle, 1978). Also, the mushroom enzyme has subunits of 41 000, 29 000, and 19 000 daltons as does RNA polymerase II from mouse plasmacytoma (Schwartz & Roeder, 1975). Interestingly, the subunits from the mushroom enzyme having molecular weights of 89 000, 69 000, and 53 000 daltons are anomolous to the general pattern for RNA polymerase II, but they are typical of RNA polymerase III for other eukaryotes (Sklar et al., 1975; Roeder, 1976; Teissere et al., 1977). It is not likely that these subunits are evident as a result of RNA polymerase III contaminating our preparation, since other subunits particular to RNA polymerase III are not found and since the data on α-amanitin sensitivity indicates the inhibition of a single RNA polymerase component by the toxin. It may very well be that the subunits of 89 000, 69 000 and 53 000 daltons from the mushroom enzyme have very little in common with the typical subunits of eukaryotic RNA polymerase III, but the question as to whether or not they are similar is testable and poses an interesting investigation. Although it is a speculative consideration, it could be that the two nucleoplasmic polymerases in eukaryotes, RNA polymerase II and III, share a catalytic core enzyme with the other more distinctive subunits possibly having a role in transcriptive specificity. With respect to this hypothesis, it does not seem unreasonable that a nucleoplasmic RNA polymerase from a lower eukaryote such as *Agaricus bisporus* may not have evolved with the evident differentiation of RNA polymerase II and III of the higher eukaryotes. Because the mushroom RNA polymerase II has a similar structural complexity with mammalian class II RNA polymerases, it can potentially be used to elucidate structure-to-function mechanisms of the RNA polymerases. Most obviously, the mushroom enzyme can be used to see whether or not subunits other than the one at 140 000 daltons have an ancillary affect in α-amanitin resistance, and quite possibly the 140 000-dalton subunit may be used in a comparative role to elucidate the functional groups involved in stabilizing the amatoxin–RNA polymerase complex.

Because of its intense staining pattern, we feel that the 99 000-dalton polypeptide may likely be a contaminant. However, the case in support of this argument is not very strong since the polypeptide is not separated from RNA polymerase by nondenaturing polyacrylamide gel electrophoresis, by glycerol-gradient centrifugation (unpublished results), or by rechromatography on either DEAE-cellulose, DEAE-Sephadex, or phosphocellulose in which the enzyme was eluted with linear salt gradients of varying slopes (unpublished results). If this polypeptide is a contaminant, it most assuredly becomes tightly associated with the enzyme complex. The possibility exists that the 99 000-dalton polypeptide is the product of proteolytic cleavage to one of the larger subunits. However, if phenylmethanesulfonyl fluoride is present in all buffers throughout the purification procedures, the subunit pattern is identical with that shown in Figure 5. If the 99 000-dalton polypeptide is the result of proteolysis, it is quite possible that the proteolytic attack is not inhibited by phenylmethanesulfonyl fluoride, which is a specific inhibitor of serine proteases. However, RNA polymerase purified through chromatography on CM-cellulose can be left at 25 °C overnight without a detectable change in subunit pattern, thus indicating the absence of proteolytic activity at least after this stage of purification. At present, we are investigating the relationship of the 99 000-dalton polypeptide to RNA polymerase II, and we expect that these results will be reported in a more detailed study on the subunit structure of the mushroom enzyme.

Although the RNA polymerase isolated from the mushroom is very insensitive to α-amanitin, the enzyme has the general characteristic properties of RNA polymerase II as reviewed by Roeder (1976), Chambon (1974, 1975), and Jacob (1973). The mushroom enzyme is quite typical of other eukaryotic RNA polymerases II. Similar to mammalian RNA polymerases II, the mushroom enzyme elutes sharply at 0.21 M $(NH_4)_2SO_4$ from DEAE-Sephadex or DEAE-cellulose. The mammalian class II polymerases as well as RNA polymerase II from the mushroom are not bound to CM-cellulose at 0.075 M $(NH_4)_2SO_4$ (Schwartz et al., 1974). The effect of ionic strength on the mushroom polymerase is rather sharply delineated with optimal activity at 0.075 M $(NH_4)_2SO_4$, which is quite typical of RNA polymerases II. RNA polymerases II generally exhibit a marked preference for Mn^{2+} over Mg^{2+} as the required metal ion. RNA polymerase II from the mushroom has a seemingly absolute preference for Mn^{2+} over Mg^{2+}. However, it should be noted that the metal titrations in this study were done with spectroscopically pure $MnCl_2$ and $MgCl_2$ and that these results may have a greater general significance when the activity of other RNA polymerases are titrated with ultrapure metals for a variety of defined templates. Like mammalian RNA polymerases II, the mushroom RNA polymerase is more active on a denatured DNA template than on a native template.

The data presented in this study indicate that RNA polymerase II from *Agaricus bisporus* is similar to mammalian RNA polymerases II while at the same time being at least 650-fold more resistant to α-amanitin. Because the enzyme can be purified in milligram quantities, it may prove to be a valuable comparative tool in the study of structure-to-function relationships for eukaryotic RNA polymerases II. Furthermore, considering the resistance to α-amanitin by RNA polymerase II from the edible mushroom *Agaricus bisporus* and the insensitivity of nuclear preparations to α-amanitin inhibition in other mushrooms (Horgen et al., 1978), it is not unreasonable to suggest that perhaps the mushrooms that synthesize amatoxins may be able to actively synthesize RNA under conditions of high concentrations of intracellular amatoxins.

Added in Proof

The 99 000-dalton polypeptide has been separated from RNA polymerase II using affinity chromatography with DNA covalently linked to CM-cellulose (Potuzak & Wintersberger, 1976). On a milligram to milligram basis nearly equal amounts of the 99 000-dalton polypeptide and RNA polymerase were recovered. However, the increase in specific activity was greater than twofold.

Acknowledgments

A special thanks to Leaver Mushroom Co., Mississauga, Ontario, for generously providing us with specimens of

Agaricus bisporus. We thank R. Yocum, Harvard University, and H. Faulstich of the Max Planck Institute, Heidelberg, West Germany, for their gifts of α-amanitin. We also thank J. Ammirati and W. R. Cummins for their helpful suggestions during the course of this study. The excellent technical assistance of E. Thompson is gratefully acknowledged.

References

Amati, P., Blasi, F., Di Porzio, U., Riccio, A., & Traboni, C. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 753.

Brady, L. R., Benedict, R. G., Tyler, V. E., Stuntz, D. E., & Malone, M. H. (1974) *Lloydia 37*, 644.

Brodner, O. G., & Wieland, T. (1976) *Biochemistry 15*, 3480.

Bryant, R. E., Adelberg, E. A., & Magee, P. T. (1977) *Biochemistry 16*, 4237.

Buchwald, M., & Ingles, C. J. (1976) *Somatic Cell Genet. 2*, 225.

Burgess, A. B., & Burgess, R. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1174.

Chambon, P. (1974) *Enzymes, 3rd Ed. 10*, 261–331.

Chambon, P. (1975) *Annu. Rev. Biochem. 44*, 613.

Chan, V. L., Whitmore, G. F., & Siminovitch, L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3119.

Chrambach, A., Reisfeld, R. A., Wyckoff, M., & Zaccari, J. (1967) *Anal. Biochem. 20*, 150.

Cochet-Meilhac, M., & Chambon, P. (1974) *Biochim. Biophys. Acta 353*, 160.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.

Dezélée, S., & Sentenac, A. (1973) *Eur. J. Biochem. 34*, 41.

Dixon, M. (1953) *Biochem. J. 55*, 170.

Dynan, W. S., Jendrisak, J. J., & Burgess, R. R. (1977) *Anal. Biochem. 79*, 181.

Faulstich, H., & Cochet-Meilhac, M. (1976) *FEBS Lett. 64*, 73.

Gebert, U., Wieland, T., & Boehringer, H. (1967) *Justus Liebigs Ann. Chem. 705*, 227.

Gérault, A., & Girre, L. (1975) *C. R. Hebd. Seances Acad. Sci., Ser. D 280*, 2841.

Gornicki, S. Z., Vuturo, S. B., West, T. V., & Weaver, R. F. (1974) *J. Biol. Chem. 249*, 1792.

Hildebrandt, A., & Sauer, H. W. (1973) *FEBS Lett. 35*, 41.

Hildebrandt, A., Sebastian, J., & Halvorson, H. O. (1973) *Nature (London), New Biol. 246*, 73.

Hjertén, S. (1962) *Arch. Biochem. Biophys., Suppl. 1*, 147.

Hodo, H. G., & Blatti, S. P. (1977) *Biochemistry 16*, 2334.

Horgen, P. A., Vaisius, A. C., & Ammirati, J. F. (1978) *Arch. Microbiol. 118*, 317.

Ingles, C. J., Guialis, A., Lam, J., & Siminovitch, L. (1976) *J. Biol. Chem. 251*, 2729.

Jacob, S. T. (1973) *Prog. Nucleic Acid Res. Mol. Biol. 13*, 93.

Jendrisak, J. J., & Burgess, R. R. (1975) *Biochemistry 14*, 4639.

Jendrisak, J., & Guilfoyle, T. J. (1978) *Biochemistry 17*, 1322.

Kedinger, C., Gniazdowski, M., Mandel, J. L., Gissinger, F., & Chambon, P. (1970) *Biochem. Biophys. Res. Commun. 38*, 165.

Kedinger, C., Gissinger, F., & Chambon, P. (1974) *Eur. J. Biochem. 44*, 421.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lindell, T. J., Winberg, F., Morris, P. W., Roeder, R. G., & Rutter, W. J. (1970) *Science 170*, 447.

Lobban, P. E., Siminovitch, L., & Ingles, C. J. (1976) *Cell 8*, 65.

Morris, P. W., Venton, D. L., & Kelley, K. M. (1978) *Biochemistry 17*, 690.

Pong, S. S., & Loomis, W. F., Jr. (1973) *J. Biol. Chem. 248*, 3933.

Potuzak, H., & Wintersberger, U. (1976) *FEBS Lett. 63*, 167.

Roeder, R. (1976) in *RNA Polymerase* (Losick, R., & Chamberlain, M., Eds.) pp 285–329, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schultz, L. D., & Hall, B. D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1029.

Schwartz, L. B., & Roeder, R. G. (1975) *J. Biol. Chem. 250*, 3221.

Schwartz, L. B., Sklar, V. E. F., Jaehning, J. A., Weinmann, R., & Roeder, R. G. (1974) *J. Biol. Chem. 249*, 5889.

Shapiro, H. L., Viñuela, E., & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun. 28*, 815.

Sklar, V. E. F., Schwartz, L. B., & Roeder, R. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 348.

Smith, S. S., & Braun, R. (1978) *Eur. J. Biochem. 82*, 309.

Somers, D. G., Pearson, M. L., & Ingles, C. J. (1975a) *J. Biol. Chem. 250*, 4825.

Somers, D. G., Pearson, M. L., & Ingles, C. J. (1975b) *Nature (London) 253*, 372.

Stirpe, F., & Fiume, L. (1967) *Biochem. J. 105*, 779.

Teissere, M., Penon, P., Azou, Y., & Ricard, J. (1977) *FEBS Lett. 82*, 77.

Tyler, V. E., & Smith, A. H. (1963) *Mycologia 55*, 358.

Vaisius, A. C., Ammirati, J. F., & Horgen, P. A. (1977) *Second Int. Mycol. Congr. Abstr., 2nd*, p 697.

Valenzuela, P., Hager, G. L., Weinberg, F., & Rutter, W. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1024.

Wieland, T., & Wieland, O. (1972) in *Microbial Toxins* (Kadis, S., Ciegler, A., & Ajl, S. J., Eds.) Vol. 8, pp 249–280, Academic Press, New York.

Wulf, E., & Bautz, L. (1976) *FEBS Lett. 69*, 6.

Young, H. A., & Whiteley, H. R. (1975) *J. Biol. Chem. 250*, 479.